The EMBO Journal vol.8 no.9 pp.2703 - 2709, 1989

Three pure chaperone proteins of *Escherichia coli* - SecB, trigger factor and GroEL - form soluble complexes with precursor proteins *in vitro*

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Communicated by G.Schatz

Diverse studies of three cytoplasmic proteins of *Escherichia coli* - SecB, trigger factor and GroEL - have suggested that they can maintain precursor proteins in a conformation which is competent for membrane translocation. These proteins have been termed 'chaperones'. Using purified chaperone proteins and precursor protein substrates, we find that each of these chaperones can stabilize proOmpA for translocation and for the translocation-ATPase. These chaperones bind to proOmpA to form isolable complexes. SecB and GroEL will also form complexes with another exported protein, prePhoE. In contrast, these chaperones do not form stable complexes with a variety of soluble proteins such as SecA protein, bovine serum albumin, ovalbumin or ribonuclease A. While chaperones may transiently interact with soluble proteins to catalyze their folding, the stable interaction between chaperones and presecretory proteins, maintaining an open conformation which is essential for translocation, may commit these proteins to the secretion pathway.

Key words: secretion/membrane assembly/leader sequences

Introduction

Since protein translocation across biological membranes is not coupled to ongoing polypeptide chain growth (Zimmermann and Meyer, 1986), the question arises as to how the newly made proteins fold prior to transit. 'Chaperone' proteins (Ellis, 1987; Hemmingsen *et al.*, 1988) were originally defined as proteins that promote proper assembly of oligomeric complexes. These proteins can also stabilize the precursor form of secreted and membrane proteins in conformations that are competent for translocation. Chaperones have been identified in both bacterial and eukaryotic translocation reactions. In mammalian systems, hsp70 can support protein transit into dog pancreas microsomes (Zimmermann *et al.*, 1988). Signal recognition particle, which binds to the amino-terminal leader sequence (Krieg *et al.*, 1986; Wiedmann *et al.*, 1987), and ribosomes, bound to the carboxy terminus of nascent chains (Perara *et al.*, 1986), may function together to prevent misfolding (Crooke *et al.*, 1988b). In yeast, four heat shock proteins, encoded by *SSA1-4*, are required for protein translocation across the endoplasmic reticulum and into the mitochondria (Deshaies *et al.*, 1988; Chirico *et al.*, 1988). In this paper, we refer to the *Escherichia coli* proteins SecB, trigger factor and GroEL as chaperones to signify their role in maintaining precursor protein competence for membrane translocation.

Chaperones are believed to participate in protein transport across the *E.coli* inner membrane (Kumamoto and Beckwith, 1985; Bochkareva *et al.*, 1988; Collier *et al.*, 1988; Crooke *et al.*, 1988a). This translocation requires a leader sequence (Gierasch, 1989; Randall and Hardy, 1989), energy in the form of ATP and the membrane electrochemical potential (Chen and Tai, 1985; Geller *et al.*, 1986), and the function of *sec* gene-encoded proteins (Emr *et al.*, 1981; Oliver and Beckwith, 1981; Kumamoto and Beckwith, 1983). Since translocation is not coupled to translation, and can only begin after the protein reaches a substantial 'critical molecular weight' (Randall, 1983), the conformation of the precursor protein is vital to the translocation process. Precursor proteins exist in an 'open' conformation prior to membrane transit, often defined operationally by the rate of digestion by proteases of low specificity (Wolfe and Wickner, 1984; Randall and Hardy, 1986; Park *et al.*, 1988). Randall and Hardy (1986) have shown that an 'open' (i.e. unfolded) conformation of pre-maltose binding protein is a prerequisite for assembly, and both the leader sequence and interactions with other proteins govern presecretory protein conformation. Since spontaneous protein folding is a very rapid event, the 'open' conformation presumably entails considerable secondary and tertiary structure, but is not the most stable structure which the protein eventually assumes after it crosses the plasma membrane. The 'open' conformation may be stabilized by chaperones (Kumamoto and Gannon, 1988; Collier *et al.*, 1988; Crooke *et al.*, 1988b).

Experiments in bacterial cell-free protein translocation reactions have suggested that several soluble cytoplasmic factors can interact with precursor proteins to stabilize them for translocation. Trigger factor was isolated due to its ability to stabilize proOmpA, the precursor form of outer membrane protein A, in an assembly competent state (Crooke and Wickner, 1987; Crooke *et al.*, 1988b). Trigger factor is a 63 kd ribosomal protein which forms a soluble 1:1 complex with proOmpA (Crooke *et al.*, 1988b; Lill *et al.*, 1988). SecB stabilizes preMBP for assembly *in vivo* and *in vitro* (Collier *et al.*, 1988; Kumamoto and Gannon, 1988; Weiss *et al.*, 1988). Deletion analysis has shown that SecB recognizes domains within the mature region of preMBP (Collier *et al.*, 1988). SecB has been purified and found to be an oligomer of 17 kd subunits (Weiss *et al.*, 1988; Kumamoto *et al.*, 1989). GroEL is a bacterial heat shock protein of 14 identical 65 kd subunits (Hendrix, 1979; Hohn *et al.*, 1979) which is involved in phage and oligomeric pro-

tein assembly (Georgopoulos and Hohn, 1978; Hemmingsen *et al.*, 1988; Goloubinoff *et al.*, 1989). GroEL binds newly made pre-β-lactamase in an *in vitro* protein synthesis reaction (Bochkareva *et al.*, 1988). This interaction stabilizes the pre-β-lactamase for membrane transit, suggesting a possible role for GroEL in protein translocation. Though studied with different assays and with different precursor proteins, these biochemical studies suggest that GroEL, trigger factor and SecB may function in a similar manner to stabilize precursor proteins for secretion. *In vivo* evidence for a role in export has only been reported for the SecB protein (Kumamoto and Beckwith, 1983).

Since much of the evidence for the roles of chaperones in translocation has been obtained under diverse *in vitro* reactions with different precursor proteins, we have compared their properties in more defined conditions with pure chaperone proteins and pure precursor substrates. We now report that each of these *E.coli* chaperones forms similar complexes with proOmpA. Complex formation stabilizes proOmpA as a substrate for the translocation ATPase and for translocation itself. This stabilization is accompanied by the formation of isolable complexes. Strikingly, these chaperones will not form such complexes with soluble proteins, suggesting an early and fundamental distinction in the assembly pathway of proteins that are targeted to a membrane.

Results

To analyze the interactions of bacterial precursor proteins and chaperones, we isolated pure precursor proteins proOmpA and prePhoE and pure chaperones trigger factor, SecB and GroEL (Figure 1). Each chaperone was assayed for its stabilization of proOmpA during the period before its insertion into inverted plasma membrane vesicles from *E.coli*. When [^{35}S]proOmpA was diluted from 8 M urea, it was able to translocate whether or not a chaperone was present (Crooke *et al.*, 1988a; Figure 2A). If the proOmpA was diluted from urea and incubated for 2 h at 20°C, it lost its competence for membrane transit (Figure 2B, lane 1; Crooke *et al.*, 1988a). In contrast, the proOmpA retained its competence for translocation if trigger factor (lane 2), SecB (lane 3) or GroEL (lane 4) was present. At equivalent molar concentrations, each chaperone was able to stabilize proOmpA to the similar degree. ProOmpA was not maintained in a competent state by DnaK, the hsp70 homolog in *E.coli*, in either the absence or presence of ATP (lanes 5 and 6).

We have recently reported the direct assay of 'translocation ATPase', defined as the hydrolysis of ATP which requires precursor proteins and is catalyzed by *sec* gene-encoded enzymes. Translocation ATPase activity requires SecA protein, membranes with functional SecY/PrlA protein and translocation-competent proOmpA (Lill *et al.*, 1989). This rapid and simple assay provides a second means of assessing the stability of precursor proteins. Upon dilution from urea, proOmpA rapidly became incompetent as a substrate for translocation ATPase, but was stabilized by either SecB or by trigger factor (Figure 3A). It is difficult to assess the activity of GroEL in this assay, because of its inherent ATPase activity. PrePhoE, another outer membrane protein precursor, also functions as a substrate for translocation ATPase, although with a higher apparent K_m

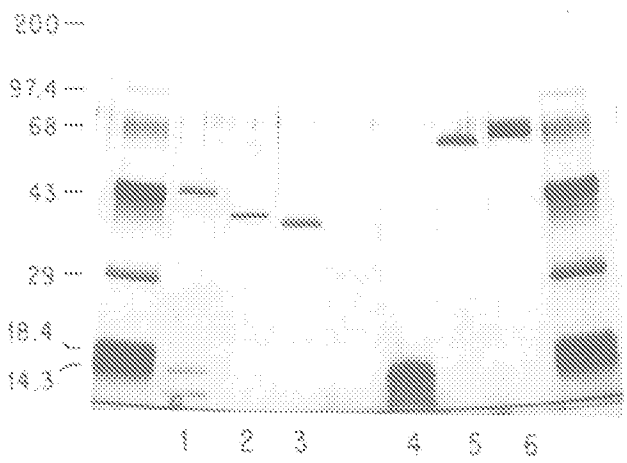

Fig. 1. SDS–PAGE of purified chaperone and precursor proteins. Each protein (500 ng) was analyzed by SDS–PAGE on 15% polyacrylamide gels containing 6 M urea (Ito *et al.*, 1980). The gel was developed by silver staining (Ansorge, 1983). **Lane 1**, prePhoE; **lane 2**, proOmpA; **lane 3**, OmpA; **lane 4**, SecB; **lane 5**, trigger factor; **lane 6**, GroEL. The mol. wts of marker proteins (myosin heavy chain, phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, β-lactoglobulin, lysosyme) are shown in kilodaltons.

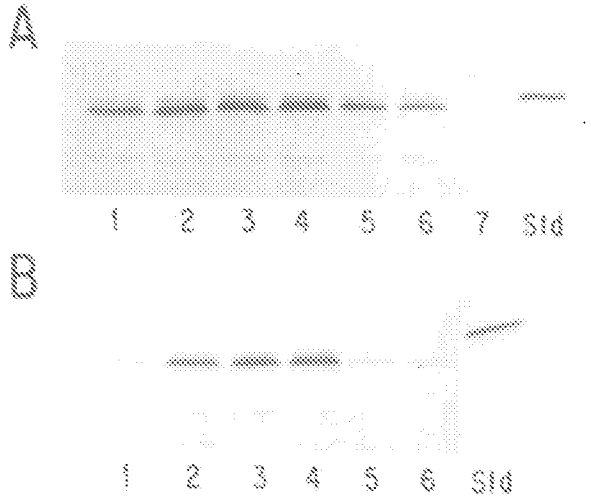

Fig. 2. SecB, trigger factor and GroEL stabilize proOmpA for membrane translocation. [^{35}S]proOmpA in buffer U (50 mM Tris–Cl, pH 8.0, 1 mM DTT, 8 M urea; 2 μl, 70 000 c.p.m./μl) was diluted into translocation mixtures (172 μl) containing no additional protein (**lane 1**), 1.75 μg/ml trigger factor (**lane 2**), 2.1 μg/ml SecB (**lane 3**), 23.3 μg/ml GroEL (**lane 4**), 1.75 μg/ml dnaK (**lane 5**) or 1.75 μg/ml dnaK and 5 mM ATP (**lane 6**). Aliquots (43 μl) were removed from each mixture immediately (**panel A**) or after 2 h at 20°C (**panel B**). Each sample was combined with 7 μl of a solution of D10 inner membrane vesicles, SecA and ATP to final concentrations of 200 μg/ml, 20 μg/ml and 5 mM respectively. Translocation reactions were incubated at 40°C for 20 min. Samples then were digested with proteinase K to reveal translocated, protected proOmpA and OmpA and analyzed by SDS–PAGE and fluorography as previously described (Ito *et al.*, 1980). Translocation reactions contained 50 mM Tris–Cl, pH 7.6, 35 mM KCl, 25 mM NH_4Cl, 10 mM $Mg(OAc)_2$, 8 mM putrescine–HCl, 1 mM spermidine–HCl, 1 mM DTT, 0.5 mg/ml bovine serum albumin. **Lane 7** on panel A contains a mock translocation reaction containing no membrane vesicles as a control for the protease treatment. During the 2 h incubation at 20°C, there was no loss of soluble radioactive proOmpA in the samples (data not shown). Standard lanes on each panel contain 25% of the amount of proOmpA used in each translocation reaction.

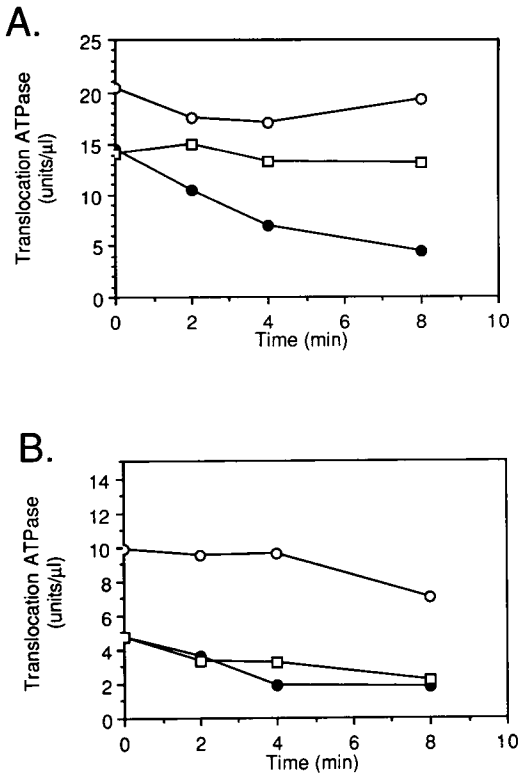

Fig. 3. Chaperones affect the ability of proOmpA and prePhoE to function as substrates for the translocation ATPase. ProOmpA (**panel A**) or prePhoE (**panel B**) in buffer U were diluted 30-fold into reaction buffer alone, or containing SecB or trigger factor. After various time intervals at 40°C, 20 μl of these mixtures were added to 5 μl of a solution containing SecA, membrane vesicles and ATP, and incubated for an additional 10 min at 40°C. The assay was performed in 50 mM Tris−Cl, pH 7.6, 30 mM KCl, 30 mM NH_4Cl, 1 mM $Mg(OAc)_2$, 1 mM DTT, 1 mg/ml bovine serum albumin and contained a final concentration of 1 mM ATP, 50 μg/ml SecA and 100 μg/ml urea washed KM9 inverted membrane vesicles. Phosphate released from ATP hydrolysis was quantitated by the method of Lanzetta *et al.* (1979). Translocation ATPase units were obtained by subtracting the units of ATP hydrolyzed in reactions lacking the precursor protein from reactions containing the precursor. 1 unit = 1 pmol P_i released/min. **Panel A**: translocation ATPase assays with proOmpA (10 μg/ml); (●) no chaperone, (□) 25.5 μg/ml trigger factor, (○) 34.4 μg/ml SecB. **Panel B**: translocation ATPase assays with prePhoE (40 μg/ml); (●) no chaperone, (□) 94 μg/ml trigger factor, (○) 127 μg/ml SecB.

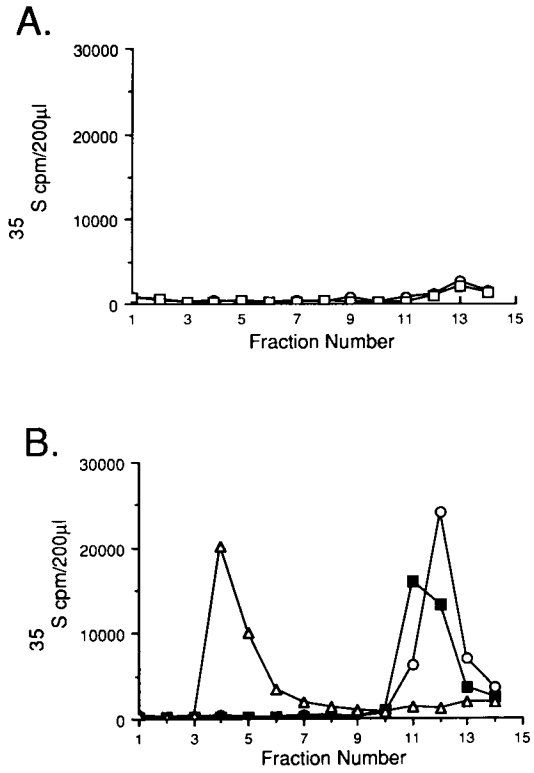

Fig. 4. ProOmpA forms soluble complexes with SecB, trigger factor and GroEL. A mixture of [^{35}S]proOmpA and proOmpA in buffer U (4 μl, 106 000 c.p.m./μl, 14.8 μg, 0.4 nmol) was rapidly diluted to 0.1 ml in solutions of various proteins in buffer G. These samples were layered onto sucrose gradients and sedimented as described in Materials and methods. Fractions (1 ml) were collected from the bottom of each gradient and analyzed by scintillation counting. **Panel A**: proOmpA mixture diluted into (○) buffer G and (□) bovine serum albumin (13.2 μg, 0.2 nmol). **Panel B**: proOmpA mixtures diluted into (■) SecB (17 μg, 0.2 nmol), (○) trigger factor (12.6 μg, 0.2 nmol) or (△) GroEL (182 μg, 0.2 nmol).

(K.Cunningham and Wickner, in preparation). SecB stimulated the activity of prePhoE in the translocation ATPase assay (Figure 3B), while trigger factor had a marginal effect on prePhoE. This agrees well with the ability of SecB, but not trigger factor, to form a stable complex with prePhoE (see below).

To explore the physical basis of chaperone stabilization, we determined whether proOmpA forms an isolable complex with SecB, trigger factor or GroEL. Aliquots of [^{35}S]-proOmpA, purified in 8 M urea, were rapidly diluted into solutions of each of the three chaperones and sedimented through linear sucrose gradients. Equimolar amounts of chaperone were used in each gradient, and the proOmpA was in a 2-fold molar excess to each chaperone. When proOmpA was diluted from urea, either without chaperone or with bovine serum albumin, it aggregated and was not recovered in the gradient (Figure 4A). However, the proOmpA was found in a soluble form when SecB, trigger factor or GroEL were included, indicating the formation of a complex between proOmpA and these chaperone proteins (Figure 4B). The sedimentation positions of proOmpA in these gradients exactly corresponded to the positions of the chaperones on stained gels (data not shown). Previous data (Crooke *et al.*, 1988b) suggest that proOmpA and trigger factor form a 1:1 stoichiometric complex. This result is reproduced in Figure 4 and extended to the associations with SecB and GroEL. The sedimentation velocities of the complexes, as well as the fact that roughly half of the input proOmpA was recovered with each chaperone, suggest specific 1:1 complexes.

We used a similar assay system to look for complex formation between chaperones and a different precursor protein. PrePhoE, the precursor form of a phosphate pore protein in the outer membrane of *E.coli*, was solubilized in 8 M urea. Portions of the protein were diluted from urea into buffer alone or into solutions of SecB, trigger factor or GroEL. These mixtures were sedimented on sucrose gradients and visualized by SDS−PAGE and silver staining (Figure 5). In the absence of a chaperone protein, prePhoE, unlike the less soluble proOmpA, was found in multiple fractions of the sucrose gradient (Figure 5A). This suggests that prePhoE only underwent partial aggregation during its sedimentation. However, when either SecB or

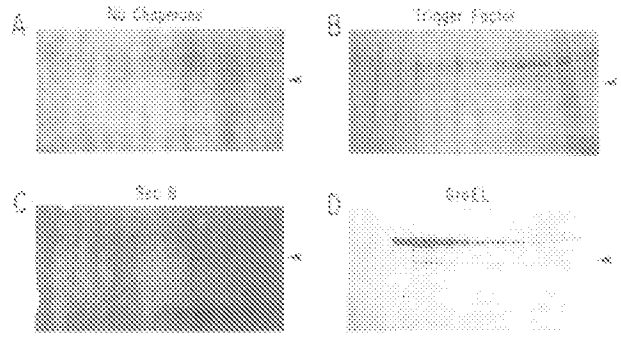

Fig. 5. GroEL and SecB, but not trigger factor, form complexes with proPhoE. PrePhoE in 8 M urea (13.3 μl, 20 μg, 0.5 nmol) was rapidly diluted into 0.2 ml in buffer G or in buffer G with one of the molecular chaperones. The samples were layered on sucrose gradients and sedimented as described in Materials and methods. Fractions (1 ml) were collected from the bottom of each gradient and 50 μl aliquots were analyzed by SDS–PAGE and silver staining. **Panel A**: no chaperone present during prePhoE dilution. **Panel B**: trigger factor (17.5 μg, 0.25 nmol) added. **Panel C**: SecB (21.3 μg, 0.25 nmol) added. **Panel D**: GroEL (235 μg, 0.25 nmol) added. In each panel, the bottom of the gradient is on the left. Arrowheads marks the mol. wt of prePhoE on SDS–PAGE.

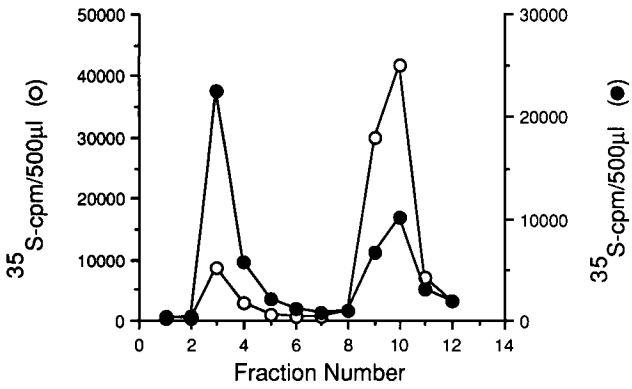

Fig. 6. ProOmpA exchanges between complexes with different chaperones. [^{35}S]ProOmpA (40 μg, 120 000 c.p.m./μl, 1.1 nmol, 20 μl) was diluted to 0.4 ml in buffer G containing SecB (46 μg, 0.54 nmol) or trigger factor (34 μg, 0.54 nmol). These mixtures were sedimented on linear sucrose gradients as described in Materials and methods. Aliquots (1 ml) were removed and the peak fractions, containing purified [^{35}S]proOmpA complexes, further manipulated. [^{35}S]ProOmpA–trigger factor complex (0.5 ml, 540 c.p.m./μl, 0.12 nmol) and [^{35}S]proOmpA–SecB complex (0.5 ml, 756 c.p.m./μl, 0.17 nmol) were diluted to 1 ml in buffer G containing GroEL (218 μg, 0.24 nmol and 309 μg, 0.34 nmol respectively). The mixtures were incubated for 5 min at 37°C and resedimented on sucrose gradients as previously described. Aliquots (0.5 ml) of each gradient fraction (1 ml, 12 fractions) were analyzed by liquid scintillation counting. (○) [^{35}S]proOmpA–SecB complex + added GroEL. (●) [^{35}S]proOmpA–trigger factor complex + added GroEL.

GroEL was present, prePhoE was only found in the fractions containing the chaperone. These results strongly suggest that prePhoE formed a specific complex with SecB or GroEL. Trigger factor did not form an isolable complex with prePhoE, in agreement with the observation that SecB, but not trigger factor, stimulated prePhoE-dependent translocation ATPase (Figure 3B).

Exchange reactions can also be used to estimate the relative affinities of the chaperones for precursor proteins. SecB appears to have a higher affinity for these precursor proteins than trigger factor. The complex of [^{35}S]proOmpA and trigger factor was isolated, then incubated with GroEL

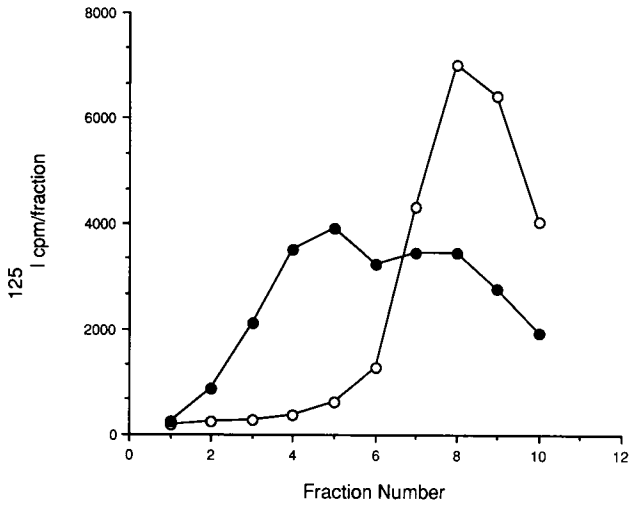

Fig. 7. [^{125}I]Trigger factor will exchange into proOmpA–trigger factor complex. FPLC-purified proOmpA–trigger factor complex (30 μl, 175 μg/ml; Crooke *et al.*, 1988b) was combined with a mixture of iodinated and unlabeled trigger factor (120 μl, 37.5 μg/ml, 500 c.p.m./μl) in buffer G and immediately reapplied to an FLPC Superose 12 10/30 (Pharmacia) sizing column. The column was eluted at 0.75 ml/min and 0.25-ml fractions were collected beginning at 11 ml after injection. The column was eluted with 50 mM Tris–Cl, pH 7.6, 25 mM NH_4Cl, 10 mM $Mg(OAc)_2$, 0.5 mM β-mercaptoethanol. Each fraction was counted in a gamma scintillation counter. The elution profiles of radiolabeled trigger factor in the absence (○) or presence (●) of purified proOmpA–trigger factor complex are shown. Note the shift of ^{125}I radioactivity from fraction 8 to the complex peak in fractions 4 and 5.

and analyzed by sedimentation. Most of the proOmpA exchanged to form a complex with the GroEL (Figure 6, filled circles). When an analogous experiment was performed starting with [^{35}S]proOmpA–SecB complex, much less exchange took place (Figure 6, open circles). As expected from these results, experiments that started with [^{35}S]-proOmpA–GroEL complex yielded almost complete exchange of proOmpA into SecB complex. However, less proOmpA was transferred to trigger factor under identical conditions (data not shown).

In additional experiments, we were able to demonstrate direct exchange of [^{125}I]trigger factor into proOmpA–trigger factor complex (Figure 7) using a gel-filtration column which separates trigger factor (fraction 8) from proOmpA–trigger factor complex (fraction 5). These results further suggest that the associations of the precursor proteins with chaperones are reversible, and that SecB forms the most stable interactions of the three chaperones.

To understand better the specificity of chaperone binding, the three chaperones were assayed for complex formation with soluble proteins. Neither SecB, GroEL nor trigger factor formed stable complexes with native SecA (Figure 8D–F). Complex formation was also not seen when SecA was denatured in 8 M urea prior to dilution into solutions containing each chaperone (Figure 8A–C). Similar experiments were carried out with bovine serum albumin, ovalbumin, lysozyme and ribonuclease A. No evidence was found of formation of a stable complex between any chaperone and any of these soluble proteins (data not shown). SecA and bovine serum albumin were chosen for their large size, which might suggest apolar internal domains in the folded protein structure. In addition, these proteins are known to interact with amphiphiles; bovine serum albumin binds fatty acids and the ATP-

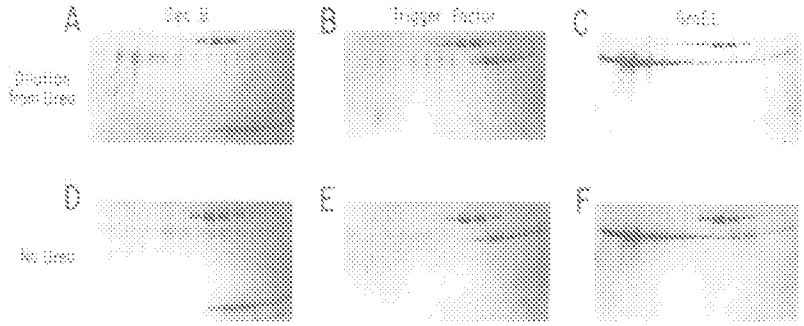

Fig. 8. SecA, a soluble protein, does not form isolable complexes with SecB, trigger factor or GroEL. SecA (5 μl, 0.8 nmol) in buffer G or buffer U (urea buffer) was rapidly diluted to 0.2 ml in solutions containing either SecB (70 μg, 0.8 nmol), trigger factor (50 μg, 0.8 nmol) or GroEL (300 μg, 0.33 nmol). The mixtures were layered on sucrose gradients and sedimented as described in Materials and methods. Fractions (1 ml) were collected from the bottom of each gradient and 50 μl aliquots were analyzed by SDS–PAGE and silver staining. **Panels A–C**, SecA diluted from urea; **panels D–F**, SecA diluted from buffer G. **Panels A** and **D**, samples with SecB; **panels B** and **E**, samples with trigger factor; **panels C** and **F**, samples with GroEL. In each panel, the bottom of the gradient is represented on the left.

ase activity of SecA protein is exquisitely sensitive to even low levels of non-ionic detergents (K.Cunningham, unpublished). Finally, in case the chaperones recognize regions other than the leader, several of these soluble proteins examined are secreted proteins. Even proteins such as bovine serum albumin and ovalbumin, which tend to aggregate upon dilution from urea, and might therefore be thought to have exposed apolar domains, did not stably associate with the chaperones (data not shown). Since precursor–chaperone associations are readily reversible (Figures 6 and 7), it is possible that these soluble proteins initially associated with the chaperones, but that intramolecular folding or our conditions of sedimentation disrupted the transient complexes. Further analysis is needed to resolve this question.

To test whether chaperones specifically recognize leader sequences, or merely apolar or amphiphilic protein domains, OmpA was isolated in 8 M urea and, as with the proOmpA and prePhoE, was diluted from the urea into a solution containing each chaperone protein. While OmpA is clearly not as soluble as SecA, bovine serum albumin or ribonuclease A, its solubility in the absence of chaperone is high enough to allow a small amount to sediment as a monomer during sucrose gradient centrifugation (Figure 9A, fractions 2 and 3 from the top). Unlike the more soluble proteins, OmpA associates with trigger factor (Figure 9B, fractions 3 and 4), SecB (Figure 9C, fractions 3 and 4) or GroEL (Figure 9D, fractions 10 and 11). Although OmpA is an integral membrane protein, nowhere in its sequence are there more than four consecutive apolar amino acid residues (Chen *et al.*, 1980). These results suggest that it is not apolarity alone that is being recognized by the chaperone.

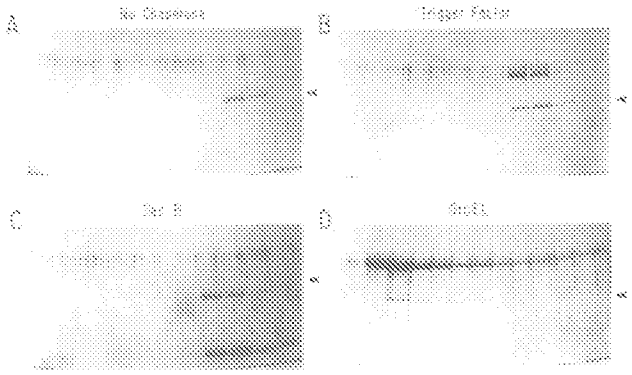

Fig. 9. OmpA forms soluble complexes with SecB, trigger factor and GroEL. OmpA in buffer U (2 μl, 10 μg, 0.29 nmol) was rapidly diluted to 0.1 ml of buffer G or buffer G containing one of the molecular chaperones. The mixtures were layered on sucrose gradients and sedimented as described in Materials and methods. Fractions (1 ml) were collected from the bottom of each gradient. Protein from 0.2 ml aliquots of each fraction was precipitated by addition of trichloroacetic acid to 10% (v/v). Precipitates were collected by centrifugation in a microfuge for 10 min, suspended in 1.0 ml cold acetone, and again collected by centrifugation. Samples were analyzed by SDS–PAGE and silver staining. **Panel A**: no chaperone present upon OmpA dilution. **Panel B**: trigger factor (9.1 μg, 0.15 nmol) added. **Panel C**: SecB (12.3 μg, 0.15 nmol) added. **Panel D**: GroEL (132 μg, 0.15 nmol) added. In each panel, the bottom of the gradient is on the left. Arrowheads mark the position of OmpA on SDS–PAGE.

Discussion

There is a striking correlation between the binding interactions and the functional interactions of each of the three chaperone proteins with proOmpA and prePhoE. Each chaperone stabilizes proOmpA for transit into inner membrane vesicles of *E.coli*; SecB (but not trigger fraction) has been found to stabilize prePhoE for this membrane transit reaction (R.Kuster, T.deVyer and B.deKruijff, personal communication). Both SecB and trigger factor stabilize proOmpA for the translocation ATPase reaction and SecB stimulates the reaction containing prePhoE as well. In a sedimentation assay, with the exception of the prePhoE and trigger factor pair, each chaperone also forms a complex with each precursor protein. These complexes are stable enough to be isolated by sucrose gradient sedimentation yet dissociate rapidly enough to permit exchange of precursor protein with competing chaperone.

The formation of the chaperone–precursor complexes was studied in the absence of ATP. This is in contrast to the heat shock protein class of molecular chaperones (Hemmingsen *et al.*, 1988). These proteins are thought to recognize and unfold misfolded polypeptides in an ATP-dependent fashion (Pelham, 1986; Rothman and Kornberg, 1986). Such ATP-dependent unfoldases have been implicated in translocation across the endoplasmic reticulum (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Zimmermann *et al.*, 1988). Since, however, precursor proteins in *E.coli* interact with

chaperones and begin translocation before folding to a mature conformation, an ATP-dependent 'unfoldase' is not obviously required (Randall and Hardy, 1989).

What is the basis of recognition, complex formation and stabilization of these precursor proteins by the chaperones? Chaperone proteins clearly do not form stable complexes with most soluble, globular proteins. Our data suggest that GroEL, trigger factor and SecB are not truly specific for leader sequences, as each will form an isolable complex with OmpA, and SecB recognizes the mature domain of the precursor of maltose binding protein (Collier *et al.*, 1988). Hydrophobicity may be one important element in binding, but further, more quantitative studies will be essential to establish the basis of this recognition.

The physiological role of each chaperone protein in bacterial protein export across the plasma membrane has yet to be fully assessed. It is quite possible that the *E.coli* chaperones have overlapping functions, as has been established for the heat-shock proteins encoded by the *SSA1–SSA4* genes of *Saccharomyces cerevisiae* (Deshaies *et al.*, 1988). Although each of these yeast proteins alone is dispensible for cell growth, as a group they are vital for protein transit into mitochondria or endoplasmic reticulum. In *E.coli*, complexes containing SecB and the precursor of maltose-binding protein have been detected in extracts of pulse-labeled wild-type cells (C.A.Kumamoto, unpublished). *In vivo* studies employing *secB* null mutations (Kumamoto and Beckwith, 1985; Collier *et al.*, 1988) have clearly shown that SecB facilitates the export of certain proteins, such as the precursors of maltose-binding protein and OmpA, but its absence has little effect on the export of others. The isolation of the genes encoding SecB (Kumamoto and Nault, 1989), GroEL (Hemmingsen *et al.*, 1988) and, more recently, trigger factor (B.Guthrie, unpublished) may allow a better evaluation of the role of each in the intact cell. To this end, we are currently attempting to engineer a strain in which the synthesis of trigger factor is governed by a tightly regulated promoter. It is also noteworthy that E.Altman and S.Emr have found that genetic induction of the heat shock response in *E.coli* will bypass the growth defects of the *secB* null mutation (E.Altman and S.Emr, personal communication).

Several hints about the relative functions of different chaperones have emerged from the biochemical studies. SecB is the most potent in stabilizing proOmpA for translocation, forms the tightest complexes and even stimulates the translocation ATPase when assayed with freshly renatured proOmpA or prePhoE (Figure 3 and S.Lecker, unpublished observations). However, due to its low abundance, it is not the major chaperone activity detected in crude extracts (Kumamoto *et al.*, 1989). A fraction of the trigger factor is bound to ribosomes (Lill *et al.*, 1988), suggesting that it might stabilize nascent chains for subsequent membrane transit.

The ability of chaperone proteins to form complexes with presecretory proteins, and inability to complex stably with globular proteins, suggests that this might serve as an early, and perhaps fundamental, distinction between cytoplasmic and exported proteins. In this model (Wickner, 1989), nascent chains of all proteins may complex with chaperones, but cytoplasmic proteins rapidly fold to their mature structure, internalizing their apolar residues and displacing chaperones. Exported proteins may be those that form a more stable complex with chaperones and thereby avoid folding to their mature structure. Our current studies reveal a correlation: two exported protein precursors (prePhoE and proOmpA) can form complexes with chaperones while several globular proteins (SecA, albumin, ovalbumin, lysozyme, ribonuclease) can not. If this correlation is generally true, then understanding the binding specificity of chaperones could reveal the fundamental distinctions between cytoplasmic and non-cytoplasmic proteins.

Materials and methods

Bacterial strains

Inverted inner membrane vesicles used in the translocation and translocation ATPase assays were prepared from *E.coli* strains D10 (Crooke *et al.*, 1988b) and KM9 (Cunningham *et al.*, 1989) respectively. The SecA overproducing strain BL21 (λDE3)/pT7SecA was described in Cabelli *et al.* (1988). The SecB overproducing strain BL21(λDE3)/pJW25 was described in Weiss *et al.* (1988). The GroEL overproducing strain OF266/pOF24 was described in Fayet *et al.* 1986). Cells were grown in 150 l cultures, harvested in a Sharples centrifuge, frozen in Tris–sucrose buffer as described (Wickner *et al.*, 1972) and stored at −80°C until use.

Materials

Urea (ultra pure) and sucrose were from Schwarz/Mann and proteinase K was from Boehringer Mannheim. β-Mercaptoethanol was from Fischer. Fatty acid free bovine serum albumin, chicken ovalbumin, ribonuclease A, dithiothreitol (DTT), and phenylmethyl sulfonyl-fluoride (PMSF) were from Sigma.

Biochemicals

Inner membrane vesicles were prepared from frozen cells following a published procedure (Chang *et al.*, 1978). Urea-treated membranes were prepared as described in Cunningham *et al.* (1989). [^{35}S]proOmpA was prepared according to Crooke *et al.* (1987). ProOmpA was isolated from cells overproducing the protein as described in Crooke *et al.* (1988b). S40 soluble cytoplasmic extracts (see SecB and GroEL isolations) were prepared as described in Crooke *et al.* (1988b). [^{125}I]Trigger factor was prepared according to the procedure of Marchalonis (1969). SecA protein was prepared as in Cunningham *et al.* (1989). PrePhoE was generously provided by Dr Ben deKruijff. Protein concentrations were assayed according to Bradford (1976), or Lowry *et al.* (1951) using bovine serum albumin as a standard.

SecB

SecB protein was prepared as described in Weiss *et al.* (1988) with minor modifications. Cells were grown, suspended and frozen as previously described (Wickner *et al.*, 1972). An S40 cytoplasmic extract was prepared from 100 g of cell suspension and applied directly to a 5 × 10 cm column of Fast Flow Q (Pharmacia) anion exchange resin. The proteins were eluted with a linear NaCl gradient of 0–600 mM (total volume 3.6 l). Fractions were assayed by SDS–PAGE and silver staining was used to detect protein. The peak fractions containing SecB were pooled and precipitated with ammonium sulfate as described (Weiss *et al.*, 1988). The redissolved protein was applied to a 1.5 × 100 cm column of S200 HR (Pharmacia) gel filtration medium. The peak fractions were pooled and dialyzed against 20 mM Tris–Cl, pH 7.4, 0.1 mM PMSF. Finally, the protein was applied to an FPLC MonoQ 10/10 (Pharmacia) column. A 150 ml 0–60 mM NaCl gradient was used to elute pure SecB at ~430 mM NaCl.

GroEL

An S40 soluble extract (Wickner, 1972) was prepared from the groEL/groES overproducer strain OF266/pOF24 (Fayet *et al.*, 1986). This extract was further clarified by ultracentrifugation for 1 h at 100 000 *g*. Aliquots (2.0 ml, 18 mg/ml) of the extract were layered on six 36 ml linear 10–30% sucrose gradients in buffer G [50 mM Tris–Cl, pH 7.6, 35 mM KCl, 25 mM NH_4Cl, 10 mM $Mg(OAc)_2$, 1 mM DTT]. The samples were sedimented in a Beckman SW27 swinging bucket rotor at 24 000 r.p.m. at 4°C for 40 h. Fifteen 1.5 ml fractions were collected from the bottom of the gradients and the remainder discarded. The GroEL protein was well resolved from the rest of the cytoplasmic proteins. The peak fractions were pooled and dialyzed into 50 mM Tris–Cl, pH 7.6, 50 mM KCl, 10% glycerol, 1 mM β-mercaptoethanol. This material was concentrated ~10-fold with Centricon concentrators (Amicon; mol. wt cutoff 10 000).

Sucrose gradient centrifugation

12.0 ml linear 10–30% sucrose gradients in buffer G [50 mM Tris–Cl, pH 7.6, 35 mM KCl, 25 mM NH_4Cl, 10 mM $Mg(OAc)_2$, 1 mM DTT]

were prepared in SW40 ultracentrifuge tubes. Sucrose solutions were pumped into the bottom of the tubes using a peristaltic pump at a rate of 1.1 ml/min. The load solution (0.2 ml unless stated otherwise) was carefully layered on top of the gradient and subjected to ultracentrifugation at 4°C for 20 h at 36 000 r.p.m. The gradients were fractionated by pumping out the contents from the bottom at a rate of 1.65 ml/min.

Acknowledgements

We thank Dr Kirsten Skarstad for the kind gift of DnaK protein and Marilyn Rice and Douglas Geissert for their expert technical assistance. This work is supported by a grant from the National Institute of General Medical Sciences. S.L. is a fellow of the Medical Scientist Training Program and R.L. is supported by a fellowship from the Deutsche Forschungsgemeinschaft.

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Received on April 25, 1989; revised on June 5, 1989